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A surface expression vector for antibody screening

(Phage gene III; fusion protein; phagemid; library; recombinant DNA)

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SUMMARY

To select specific antibodies (Ab) from large recombinant libraries using small amounts of antigen, we have constructed a phagemid that expresses a single-chain Ab fused to pIII, a coliphage protein product of gene *III* that initiates infection by binding to F pili. Surprisingly, the production of the fusion protein (Ab::pIII) was induced by wild-type (wt) phage fd in the absence of IPTG. Ab::pIII was identified by a monoclonal Ab to an epitope in the linker sequence between the heavy and light chains, and by antisera to their N-terminal sequences. It is able to bind antigen and be assembled into infectious phagemid particles that can be enriched on columns of immobilised antigen. The phagemid DNA is even smaller than that of wt fd phages and can easily be propagated in plasmid form. Most importantly, its Ab::pIII-encoding gene can be tightly repressed so that Ab libraries can be amplified without risk of being dominated by deletion mutants. After induction, however, large quantities of the fusion protein can be produced, thus greatly facilitating its analysis.

INTRODUCTION

Plasmid and phage Ab libraries have been established in *Escherichia coli* from PCR-amplified immunoglobulin families following immunisation. Immunogen-reactive recombinant Ab were selected by an ELISA of the bacterial supernatant from isolated bacterial colonies (Ward et al., 1989) or by screening nitrocellulose plaque lift-offs of bacterial colonies for reactivity to the radioactively labeled immunogen (Huse et al., 1989; Caton and Koprowski, 1990; Mullinax et al., 1990). However, for the selection of specific Ab from randomly combined light and heavy chain libraries of nonimmunised animals that do not contain a preponderance of Ab to a particular antigen, a procedure is required for screening millions of Ab-producing bacteria.

An elegant solution to this problem would be to attach recombinant Ab to the surface of bacteria or bacteriophages so that they could then be rapidly selected by antigens bound to a solid phase. Given the difficulties of targeting proteins to the cell surface of bacteria, an attractive candidate in view of its small size and relatively simple genetic make-up is the M13 family of filamentous bacteriophages

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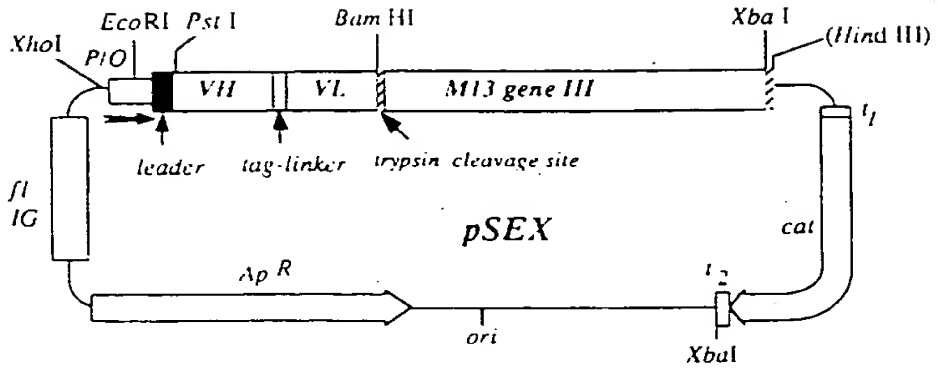
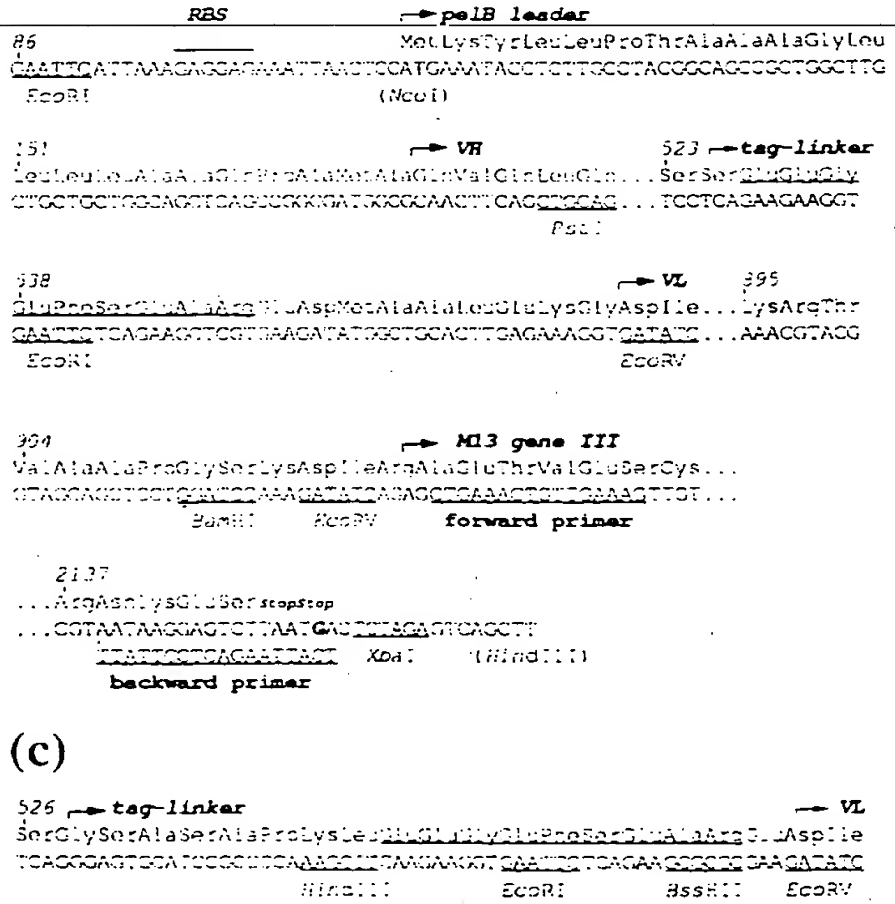
Abbreviations: aa, amino acid(s); Ab, antibody(ies); *Ab::III*, gene fusion encoding Ab::pIII fusion protein; Ap, ampicillin; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; mAb, monoclonal Ab; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; pIII, gene *III* product of phage M13; PBS, phosphate-buffered saline (150 mM NaCl/50 mM NaH_2PO_4, pH 7.4); PCR, polymerase chain reaction; pfu, plaque-forming unit(s); Polk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance/resistant; RBS, ribosome-binding site; scAb, single chain Ab; Tc, tetracycline; wt, wild type; [ ], denotes plasmid-carrier state; ::, novel joint (fusion).

(a)



(b)



(c)

Fig. 1. pSEX, a surface expression phagemid for Ab screening. *P/O*, promoter/operator; *leader*, signal peptide sequence of bacterial pectate lyase; *VH* and *VL*, heavy and light chain variable domains, respectively, of an anti-lysozyme Ab; *tag-linker*, oligo encoding 18 aa linking *VH* and *VL* that include the epitope of the mAB YOL1/34; *trypsin cleavage site*, LysAspIleArg. This optional sequence permits proteolytic cleavage of the antibody domains from pIII; *cat*, chloramphenicol acetyl transferase gene; t_1 and t_2, transcription terminators; *ori*, origin of DNA replication for ColE1; *fl IG*, intergenic region of phage f1. (a) **Construction.** To provide the necessary restriction sites, the oligos 5'-GCTGAATTCGGATCCATAGGGCCCTCTAGAGTCGAC and 5'-AATTGTCGACTCTAGAGGGCCCTATGGATCCGAATTCAGCTGCA were 5' phoshorylated, hybridised and ligated to pUC119 that had been cleaved with *Pst*I + *Eco*RI and dephosphorylated. In an optional step to create a protease-sensitive sequence, the hybridised oligos 5'-GATCCAAAGAT-ATCAGAGGGCC and 5'-CTCTGATATCTTTG were inserted between the *Bam*HI and *Apa*I sites of the first set of oligos. scAb-DNA was then inserted between the *Pst*I and *Bam*HI sites followed by the blunt-end-ligation of gene *III* DNA after cleaving the phagemid with *Apa*I and treating with T4 DNA polymerase to remove 3' overhanging ends. Phagemid pSEX was constructed by combining the multiple cloning site of pUHE24-2 with the closely related phagemid pDS31-1 that contains an additional f1 intergenic region (IG) (Bujard et al., 1987; Müller, 1989). The pDS31-1 sequence

(for reviews see Webster and Lopez, 1985; Day et al., 1988; Smith, 1988). Their DNA is enclosed in a tubular array of (approx. 2700) coat proteins encoded by gene *VIII* (Newman et al., 1977). In addition, about five copies each of gene products VII and IX are present at one end of the phage and about five copies each of gene products III and VI at the other end (Simons et al., 1981; Grant et al., 1981).

Protein pIII is a relatively flexible and accessible molecule composed of two functional domains: an N-terminal domain that binds to the F pilus of male bacteria during infection and a C-terminal domain buried within the virion that is important for morphogenesis (Gray et al., 1981; Armstrong et al., 1981; Crissman and Smith, 1984). Peptides can be inserted between the two domains of pIII (Smith, 1985) or near the N terminus (Parmley and Smith, 1988) without destroying its functions in morphogenesis and infection. After much pioneering work on the use of pIII in fd phages for carrying foreign peptides, Parmley and Smith (1988) showed that peptide epitopes inserted at the N-terminal end could bind phages to immobilised antibodies. As a direct consequence of this work it has been possible to generate peptide libraries that can be easily screened for binding to ligands and antibodies (Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990).

In an analogous approach, Ab have been attached to the surface of fd phages by inserting Ab DNA into the 5′ end of gene *III* (McCafferty et al., 1990). Alternatively, DNA coding for Ab-pIII could be incorporated into a phagemid. A major advantage of this latter system would be the ability to propagate the Ab-DNA in plasmid form under conditions where its expression is tightly repressed. The risk of Ab libraries being dominated by deletion mutants would then be greatly reduced. Fusion phage, for example, have been shown to be mainly useful for displaying relatively small inserts, probably because the larger inserts have an adverse effect on the infectivity function of pIII (Parmley and Smith, 1988). Furthermore, Ab::pIII can be produced in much higher amounts than in the phage system, thereby facilitating the analysis of isolated clones. The smaller phagemids should also be able to transform *E. coli* with higher efficiencies than phage DNA and might be expected to dissociate less easily from immobilised antigens. Here, we describe a phagemid system for the surface expression of a functional scAb.

RESULTS AND DISCUSSION

(a) Construction of surface expression vector (pSEX)

DNA coding for a scAb and pIII were cloned into pUC119 after insertion of a specific set of restriction sites and a protease-sensitive joining sequence into the multiple cloning site. The *Ab* DNA coded for the heavy and light chain variable domains of a humanised Ab against hen egg-white lysozyme (a kind gift of Dr. G. Winter) derived from the anti-lysozyme Ab D1.3 (Amit et al., 1986; Verhoeyen et al., 1988). These domains were joined by an 18-aa linker sequence containing the epitope for the mAb YOL1/34 (Breitling and Little, 1986), thus enabling the Ab to be identified. To provide a more flexible junction to pIII, the 3′ end of the light chain DNA was modified by the addition of nt coding for the first 6 aa on the human κ constant domain followed by a *Bam*HI restriction site. Gene *III* DNA was amplified from the bacteriophage M13 using primers corresponding to the 5′ and 3′ ends of gene *III*. The Ab::pIII-encoding DNA was then cloned into a phagemid of the pDS family that contains a coliphage T7 promoter combined with two *lac* operators (Bujard et al., 1987; Lanzer and Bujard, 1988; Müller, 1989). In a final step, DNA coding for the leader sequence of the bacterial enzyme pectate lyase was ligated to the 5′ end of the Ab-encoding DNA resulting in the phagemid pSEX (Fig. 1a). The leader, linker and PCR primer sequences are shown in Fig. 1b. An alternative linker sequence (Fig. 1c) with the YOL1/34 epitope placed at the end of the linker containing a useful restriction site for the insertion of Ab libraries was also employed. Although both of these tag-linkers contain a significant number of acidic aa residues, they appeared to have no effect on the production of functional scAb when compared to scAb with linkers composed only of the neutral aa, Gly and Ser (data not shown).

extends from *Xho*I anticlockwise to a *Hind*III site (in parentheses) that was lost after a blunt-end ligation. Plasmid pUHE24-2 is essentially identical to pDS6 (Bujard et al., 1987) with a coliphage T7 promoter combined with two *lac* operators and an RBS (PA1/04/03, Lanzer and Bujard, 1988; Lanzer, 1988). The resulting phagemid was cleaved with *Hind*III and the 5′ overhanging ends were filled-in with PolIk. After a further digestion with *Pst*I, the *Pst*I-*Hin*cII *Ab-III* DNA fragment was inserted into the phagemid. In a final step, synthetic DNA coding for the leader sequence of the bacterial enzyme pectate lyase and for the first four aa of the heavy chain was inserted between the *Nco*I and *Pst*I restriction sites. Plasmids pUHE were propagated in *E. coli* 71-18[pDM1] that expresses *lac* repressor; pUC plasmids were propagated in DH5α, and the Ab::pIII fusion protein was produced in JM101. (b) Sequence of the *RBS*, signal peptide sequence of the bacterial *pectate lyase* (*pelB leader*) in *Erwinia carotovora*, *tag*-linker and the PCR primers that were used to amplify gene *III* from phage M13. Underlined aa indicate the α-tubulin epitope for mAb YOL1/34. Subsequent aa in the linker sequence are a continuation of the sequence in α-tubulin after the epitope. Nucleotides are numbered starting from the *Xho*I site. (c) Alternative tag-linker sequence containing a useful restriction site (*Hind*III) for the insertion of *Ab* libraries. Underlined aa indicate the α-tubulin epitope for mAb YOL1/34. Preceding aa in the linker sequence are a continuation of the heavy chain *Ab* sequence from the end of the variable domain into the constant domain. In contrast to Fig. 1b, the α-tubulin epitope is at the end of the linker sequence. Neither of the linkers appeared to have any effect on the production of scAb when compared to linkers composed of only the neutral aa Ser and Gly.

(b) Synthesis of the Ab-pIII fusion protein

To test whether the completed phagemid vector was able to express the full-length fusion protein, 100 μM IPTG was added to a log-phase culture of *E. coli* [pSEX]. The culture showed a marked decline in its growth rate compared to the control indicating a significant synthesis of phagemid-encoded protein. On Western-blot analysis, Ab::pIII was identified by three Ab, a monoclonal antibody to part of the linker sequence (EEGEFSEAR) and two anti-peptide rabbit sera against N-terminal sequences of the heavy and light chains. It migrated as a 93-kDa protein (Fig. 2). The large size of the fusion protein (predicted M_r 68 100) is most probably due to the pIII component (M_r 42 100) that migrates as a 55–70-kDa protein (Goldsmith and Konigsberg, 1977). Partial proteolysis of the fusion protein was indicated by the presence of some minor bands of lower M_r that were identically stained by all three Ab.

Cell fractionation showed that the protein was present in the cytoplasmic and membrane fractions but not in the periplasm and culture supernatant (Fig. 2, lanes 3–6) in contrast to the Ab component alone without pIII that was secreted into the periplasm and medium (data not shown). This was not surprising since pIII is assembled onto phage particles from the inner bacterial membrane, a process that appears to be dependent only on the C-terminal domain. Deletion mutants of pIII without this domain pass into the periplasm without becoming attached to the cytoplasmic membrane (Boeke and Model, 1982) and normal phage particles are not assembled (Crissman and Smith, 1984). The anchor sequence is probably a hydrophobic stretch of 23 aa at the C terminus (Davis et al., 1985).

The ability of the fusion protein to bind antigen was investigated by passing the Triton X-100-soluble fraction over a column of lysozyme bound to sepharose. Western blots of the unbound material and the fractions obtained after thoroughly washing and eluting with 0.05 M diethylamine showed that the full-length fusion protein was indeed specifically retained on the lysozyme column (Fig. 2, lanes 7–12).

(c) Packaging of the pSEX phagemid

To determine whether the phagemid expression vector could be packaged, *E. coli* [pSEX] were multiply infected with phage fd. IPTG was not added since it was found to have an inhibitory effect on phagemid packaging. A similar finding was recently reported by Bass et al. (1990) who constructed a phagemid that expresses a fusion protein of human growth hormone and the C-terminal domain of pIII. Examination of Ab::pIII production with and without IPTG after adding phage fd showed that the phage alone was able to induce expression (Fig. 3). A possible explanation is that one of the phage gene products interferes with the binding of *lac* repressor to the operator. Alternatively,

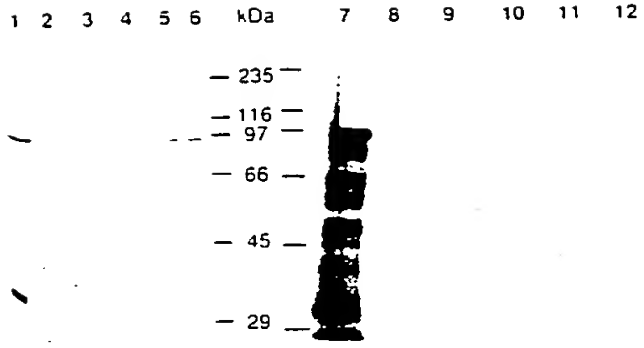


Fig. 2. Inducibility, cellular localisation and antigen binding of the Ab::pIII fusion protein analysed by gel electrophoresis on 0.1% SDS 8% polyacrylamide gels and Western blotting. Lanes: 1 and 2, total cells after 1 h induction with 100 μM IPTG (1) or without IPTG (2). Lanes: 3–6, cell fractionation: 3, culture supernatant; 4, periplasmic enriched fraction; 5, soluble cytoplasmic fraction; 6, 1% Triton X-100 extract. Lanes: 7–12, lysozyme affinity chromatography of the 1% Triton X-100 extract from induced and noninduced cells: 7, effluent (+IPTG); 8, effluent (−IPTG); 9, last wash (+IPTG); 10, last wash (−IPTG); 11, eluate (+IPTG); 12, eluate (−IPTG). Lanes 1–6 were stained using the mAb YOL1/34 (Kilmartin, 1982; Serotec, Oxford, U.K.) and lanes 7–12 using an antiserum to the N-terminal sequence of the light chain. **Methods.** Ab to heavy and light chains were obtained by subcutaneous injection of rabbits with the N-terminal peptides QVQLQQSGGGAC and DIQMTQSPSSAC, respectively, coupled to keyhole limpet haemocyanin. To investigate the expression of the fusion protein, the pelleted bacteria of IPTG-induced cultures were resuspended in 30 mM Tris·HCl pH 8.0, containing 20% sucrose, 1 mM EDTA, 1 mg per ml chick lysozyme and incubated for 10 min on ice. After centrifuging for 1 min at 12000 × *g*, the supernatant containing the periplasmic proteins was collected and the pellet was sonicated briefly in 0.1 M Tris·HCl pH 8.0. The soluble cytosolic fraction was decanted after centrifuging for 5 min at 12000 × *g* and the resuspended pellet was incubated in 1% Triton X-100 to obtain the membrane-bound fraction. All the fractions were analysed for β-lactamase activity according to Plückthun and Knowles (1987) to check the efficiency of the fractionation procedure. The Triton-soluble fraction was diluted 100-fold with PBS before applying to affinity columns. For affinity chromatography, chick lysozyme was coupled to CNBr-activated Sepharose (Pharmacia) according to the instructions of the manufacturer. The lysozyme-Sepharose was incubated for 20 min at room temperature with the extracts and poured into columns that were subsequently washed with ten bed volumes of PBS, followed by 1 M NaCl, and 0.5 M NaCl in 0.1 M $NaHCO_3$ at pH 8.3, before eluting with 0.05 M diethylamine. All the fractions were precipitated with trichloroacetic acid (final concentration 20%) and resolved on 0.1% SDS–8% PAGE (Laemmli, 1970). Western blots were performed according to Towbin et al. (1979) using second Ab coupled to horseradish peroxidase with diaminobenzidene as substrate.

phage proteins binding to the intergenic region might affect the topology of the phagemid and cause the release of the *lac* repressor. Whatever the reason, we have found that moving the intergenic region 10^3 nt to the other side of the *bla* gene has no effect on this phenomenon (data not shown).

Agarose gel electrophoresis of the DNA from virus particles secreted into the medium showed, in addition to the

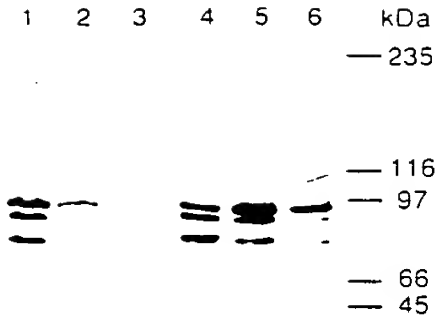


Fig. 3. Induction of Ab::pIII by infection with wt fd phage. A logarithmic culture of *E. coli* JM101[pSEX] was incubated for 3 h with or without IPTG or phage fd, respectively. Gel electrophoresis and Western blotting of the total bacterial protein were performed as described in Fig. 2. Ab::pIII was detected using the anti-heavy chain serum. Lanes: 1–3, without fd; 4–6 in the presence of fd phage (with a multiplicity of infection of 50); 1,4, 10 μM IPTG; 2,5, 4 μM IPTG; 3,6, without IPTG.

single-stranded DNA of fd, a larger quantity of smaller DNA that was compatible in size with single-stranded pSEX (Fig. 4). Further proof of phagemid packaging and

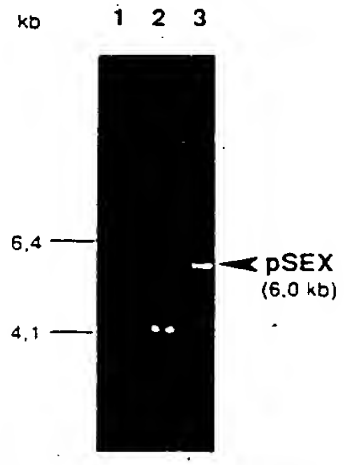


Fig. 4. Gel electrophoresis of circular single-stranded pSEX phagemid. Lanes: 1, fd; 2, control phagemid pUHE31-1 with fd; 3, pSEX with fd. **Methods.** DNA from fd virions and packaged phagemid particles was applied to 0.8% agarose gels in 0.09 M Tris/0.09 M borate/1 mM EDTA pH 8.0 according to Sambrook et al. (1989) and stained with ethidium bromide. For the preparation of packaged phagemids, *E. coli* JM101[pSEX] was plated onto M9 minimal medium (Sambrook et al., 1989) and incubated for 30 h at 37°C. 2 ml of the same medium was inoculated with one of the colonies and incubated at 37°C with vigorous aeration until it reached an A_{578nm} about 0.2. LB medium (0.5 ml; Sambrook et al., 1989) and a tenfold excess of phage fd were then added to the culture and it was incubated for a further 3 h at 37°C. After carefully centrifuging twice at 12000 × *g* for 5 min at room temperature, the supernatant was adjusted to a final concentration of 4% polyethylene glycol (Serva PEG 6000)/0.5 M NaCl and left to stand overnight at 4°C. The phagemids were sedimented by centrifuging at 12000 × *g* for 20 min at room temperature and suspended in 200 μl of a Tris · EDTA buffer pH 7.5. Phagemid DNA was prepared by shaking with 1 vol. of phenol for 10 min followed by treatment with chloroform-isopropanol and precipitated with isopropanol (Sambrook et al., 1989).

the production of infectious particles was shown by infecting *E. coli* with the secreted virus particles. 10^{10} Ap^R colonies/ml of *E. coli* were obtained compared to 3×10^7 pfu. Moreover, after growing one of the Ap^R colonies in liquid culture, the addition of IPTG induced the synthesis of Ab::pIII (data not shown).

To determine whether the packaged phagemid had incorporated the Ab::pIII fusion protein, 90 μl culture supernatant containing 5×10^8 packaged phagemids determined as Ap^R transducing units was mixed with a 1000-fold excess of wt fd phage and passed over a column of immobilised lysozyme. After thoroughly washing with ten bed volumes each of PBS/1 M NaCl and 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3, the phagemid particles were eluted with 0.05 M diethylamine. The eluate was neutralised with 0.5 M NaH_2PO_4 and assayed for the number of phages and packaged phagemids (Table I). A specific enrichment of up to 121-fold was achieved, thus demonstrating the incorporation of functional Ab::pIII fusion proteins into phagemid particles. Bass et al. (1990; see above) obtained significantly higher enrichments of phagemid particles containing human growth hormone fused to the C-terminal domain of pIII after passage over immobilised receptors, most likely due to the higher affinity of hormones and receptors. The binding properties of the Ab-phagemid particles, however, might be further increased by using a gene *III* deletion mutant phage for packaging. This would ensure that only those phagemids coding for functional fusion proteins would be packaged and all five pIII molecules on a phagemid particle would be fused to antibodies.

McCafferty et al. (1990) reported the assembly of an Ab::pIII fusion protein into an fd Tc^R phage after inserting *Ab* DNA into the 5′ end of gene *III*. The phage remained infectious and could be enriched by affinity chromatography. A major advantage of the phagemid system described here, however, is that it can be propagated as a plasmid and is not under any selection pressure to remove *Ab* DNA, since the expression of the fusion protein is tightly repressed. This is particularly important during the amplification of *Ab* libraries when faster proliferating deletion mutants could quickly dominate. The phagemid DNA, being about half the size of the above phage DNA, should also transform bacteria more efficiently. Moreover, in contrast to the above-mentioned phage system, large quantities of the smaller phagemid DNA are produced and large amounts of antibody protein are available after induction, thereby greatly facilitating its analysis.

Expression of *Ab::III* using pSEX and its packaging into viral particles should greatly facilitate the establishment of bacterial systems for the isolation of high affinity Ab. Millions of Ab-producing clones from *Ab* libraries can now be rapidly screened by binding to immobilised antigen. A further advantage over conventional screening methods is

TABLE I

Specific enrichment of packaged phagemids on an antigen affinity column

Phagemid particles + phages[a]	Total volume[b] (ml)	Ap^R colonies[c]			Total pfu[c]			Excess[f] pfu.Ap^R	Enrichment factor[g]
		Plated volume[d] (μl)	Number	Total	Plated volume[d] (μl)	Number	Total		
Applied	10	10^{-3}	51	5.1×10^{4}	10^{-6}	45	4.5×10^{11}	882	—
Eluted	1.1	10^{-1}	26	2.9×10^{3}	10^{-3}	19	2.1×10^{8}	7.3	121

[a] Phagemids mixed with a 1000-fold excess of wt fd phages.
[b] Volume of sample applied to a column of immobilised lysozyme and the volume eluted with 0.05 M diethylamine.
[c] Number of *E. coli* colonies resistant to Ap after infection with phagemid particles from the applied and eluted samples.
[d] Samples were diluted and plated such that the given amounts of original sample were present per plate.
[e] Number of pfu on a lawn of *E. coli* after infection with phagemid and phage particles from the applied and eluted samples.
[f] Ratio of the total pfu (phagemids + phages) in the applied and eluted samples to the total number of Ap^R colonies (phagemids).
[g] Enrichment of phagemid particles over phage in the eluted sample.

that only small amounts of antigen are required, an important factor when the supply of a rare protein is limited. This system also offers the possibility of screening randomly mutated Ab to increase their binding affinities. The procedure could be repeated many times until the desired specificity is achieved. It should now be feasible for the first time to carry out large-scale differential screening analyses of related cells and organisms. A subtractive selection, for example, using normal and neoplastic cells could be used to identify tumour-associated antigens. The phagemid system might also prove to be very useful for investigating the components of molecular interactions, e.g., by selecting Ab that inhibit ligand receptor binding.

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